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Article

Phytochemicals, Antioxidant, and Antidiabetic Effects of *Ranunculus hirtellus* Aerial Parts and Roots: Methanol and Aqueous Extracts

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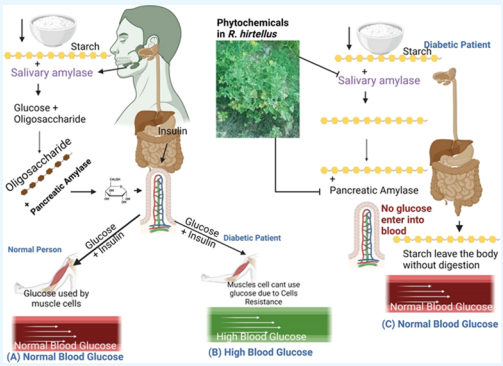
ABSTRACT: *Ranunculus hirtellus*, also known as crowfoot (buttercup), has a rich tradition of use in various biological contexts. While antibacterial studies on extracts from this plant have been conducted, the phytochemical composition, antioxidant properties, and antidiabetic effects remain unexplored. In this study, the phytochemical, antioxidant, and antidiabetic effects of its methanol and aqueous extracts were investigated. Our approach involved gas chromatography−mass spectrometry (GC/MS), alongside quantitative and qualitative methods, for phytochemical profiles. Additionally, concerning biological activities, the antioxidant effect was assessed through 2, 2-diphenyl-pieryl hydrazyl (DPPH) and 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) assays, while the antidiabetic effect was examined through the α-amylase inhibitory assay. The chloroform, ethyl acetate, and *n*-hexane extracts of *R. hirtellus* revealed the presence of 14 distinct compounds. In the methanol extract, sterols, quinones, glycosides, lactones, lignin, and flavonoids were identified. The aqueous extract contained sterols, alkaloids, glycosides, triterpenes, terpenoids, quinones, leucoanthocyanins, and lactones. The total flavonoid content (TFC), total phenolic content (TPC), total tannin content (TTC), and reducing sugar content (RDC) were determined in plant extracts, and a linear relationship was found between these parameters. Additionally, the TTC, TPC, and TFC values for both extracts hovered around 0.3786, 0.0476, and 0.1864 μg/mL, respectively, across all plant concentrations, while RDC ranged from 0.9336 to 1.0119 μg/mL in all four extracts. In vitro assays demonstrated dose-dependent antidiabetic activity in both methanolic and aqueous extracts by inhibiting α-amylase. Furthermore, the antioxidant activity observed in the DPPH assay was greater in the aqueous extract compared with the methanolic extract. In addition, the ethyl acetate extract exhibited the highest inhibition among chloroform and *n*-hexane in the ABTS assay. The results suggest that *R. hirtellus* can be a potential source of natural antioxidants and antidiabetic agents, and further studies are warranted to investigate the underlying mechanisms of its therapeutic effects.



INTRODUCTION

Diabetes mellitus (DM) is a metabolic condition distinguished by increased levels of glucose in the bloodstream and manifests in two main types.[1] Type 1 DM arises from insufficient insulin secretion due to an autoimmune response,[2] while Type 2 DM results from the body's impaired capacity to efficiently utilize insulin due to cells' resistance to insulin.[3] Untreated diabetes can exert wide-ranging effects on the body, contributing to various complications. Globally, diabetes prevalence is increasing, with an estimated 4.4% projected by 2030.[4] α-Amylase, a pivotal enzyme in carbohydrate digestion, plays a crucial role in the regulation of blood glucose levels. A plethora of research is exploring the potential of α-amylase inhibitors to manage hyperglycemia by delaying carbohydrate digestion in the gastrointestinal tract (GIT).[5] However, synthetic inhibitors have been associated with side effects,[6] underscoring the need for safe and effective alternatives.

The free-radical-induced oxidative stress is a recognized contributor to degenerative diseases.[7] This phenomenon contributes to the development of diabetes mellitus (DM) and a multitude of other health conditions, impacting metabolic processes and overall well-being.[8] Therefore, oxidative stress remains a therapeutic target to manage various diseases, including diabetes. Antioxidants play a pivotal role in defending the body against different diseases. They regulate free radicals and oxidative reactions, forming an integral part of the body's defense mechanisms.[7] This includes protection against various diseases, including cancer[9] and diabetes.[10]

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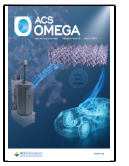

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Natural products with α-amylase inhibitory and antioxidant properties have gained attention in diabetes research[11] due to fewer side effects and easy availability.[9] Modern medicines are often derived from medicinal plants,[12] which have been used globally for their hypoglycemic[13] and antioxidant properties[14,4] to cure different diseases[3,15,16] through different mechanisms.[17,16,18] *Ranunculus*, a genus comprising approximately 600 plant species within the *Ranunculaceae* family, thrives in diverse environments from pastures and meadows to low-lying wetlands and cold alpine mountains.[19] In Pakistan, this family boasts 28 species.[20] The genus exhibits remarkable morphological and physiological variability, reflecting its strong adaptability.[20] In China, eight *Ranunculus* species are used in traditional folk medicine.[20] *Ranunculus hirtellus* Royle is a member of this family and is restricted to temperate and subalpine altitudes,[21] altitude ranges from 800 to 3000 m. *R. hirtellus* Royle is a perennial, erect, or decumbent herb,[22] and its flowers are solitary or several, yellow.[23] *R. hirtellus* Royle is particularly pubescent, having fibrous and fusiform roots that are endemic to the Himalayas.[24] It is distributed in the northeast Himalayas, spanning temperate, subalpine, and alpine slopes.[24] The plant is found at elevations ranging from 2000 to 4500 m.[24] Its habitat extends across Jammu and Kashmir states, Uttarakhand, Sikkim, Himachal Pradesh, and Arunachal Pradesh.[24] Additionally, it is also present in Nepal, Tibet, Pakistan, and Afghanistan.[24] *R. hirtellus* has a fascinating array of traditional uses including cure of testes swelling,[25] anthelminthics,[26] antiaging agents,[27] antigout agents,[28] and antimicrobials.[25] It even aids in wound healing[29] and serves as livestock feed.[30] Its traditional use against diabetes and oxidation has not been reported. Additionally, its phytochemical, antioxidant, and antidiabetic potential remain unexplored.

Although several medicinal plants possess antidiabetic effects via the reduction of oxidative stress and inhibition of α-amylase, the plant *R. hirtellus* remains relatively unexplored in this context. Thus, this is the first study that aims to investigate phytochemical constituents through GCMS analysis and qualitative and quantitative tests. Additionally, the aim of this study was also to explore the antidiabetic effect of different extracts through α-amylase inhibition and antioxidant effect through DPPH and ABT inhibition.

MATERIALS AND METHODS

Chemical and Reagents. Ethyl acetate, chloroform, methanol, ethylene, *n*-hexane, gallic acid, sodium hydroxide, dimethyl sulfoxide, acarbose, buffer solution, potassium sodium tartrate, DNS (3,5-dinitrosalicylic acid), amylase (aspergillus), etc. were used in the experiments. Majority of the reagents were purchased from Sigma-Alrdich (Steinheim, Germany).

Plant Selection and Extraction. *Plant Selection.* The study utilized healthy *R. hirtellus* plants that were collected from various areas of Tehsil Sherwan, District Abbottabad, Khyber Pakhtunkhwa, Pakistan. Sherwan is situated 35 km west of Abbottabad (34° 12′ 0″ North, 73° 4′ 0″ East) at a height of 1194 m. The plant specimens were authenticated by expert botanists, and the stems and leaves underwent extensive washing with water to eliminate any contaminants. Afterward, they were air-dried in the shade for approximately 6 weeks (1.5 months) and then ground into a fine powder using a grinder.

Filtration and Extract Preparation. Extract preparation was carried out from ariel parts and roots by the procedure described in ref 31. As per the procedure, shade-dried materials were ground into powder, and a total of 200 g was immersed in 500 mL of solvent mixture for 05 days. The powder and solvent (double the required concentration) ratios are as follows: methanol (50 g/250 mL or 1:5), aqueous (50 g/250 mL or 1:5), ethyl acetate (25 g/125 mL or 1:5), chloroform (25 g/125 mL or 1:5), and *n*-hexane (25 g/125 mL or 1:5). After soaking, Whatman No. 1 paper was used to filter the extract. The filtrate was then heated to 40 °C and run through a rotary evaporator under low pressure. These dried crude extracts were labeled and stored at 4 °C for further studies.

Phytochemical Analysis. *Gas Chromatography–Mass Spectrometry.* GC/MS analysis was conducted following Thangavel et al., method with modifications.[32] For GC/MS, powdered *R. hirtellus* (200 g) was mixed with 500 mL of solvent. To prepare the samples, 20 g of *R. hirtellus* powders (aerial parts and roots) were used separately. They were soaked in 50 mL of ethyl acetate, chloroform, and *n*-hexane, respectively. The ratio used was 200 g of plant material to 500 mL of solvent. The 1.5 mL filtrate was taken after 5 days of soaking in these solvents. GC/MS analysis utilized a Thermo GC-Trace ultraversion 5.0 instrument coupled with a Thermo MS DSQ II Mass Spectrometer. The samples were separated into a DB5-MS capillary column (30 m × 0.25 mm × 0.25 μm) using helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature was gradually raised from 70 to 260 °C at a rate of 6 °C/min, with a total running time of 43 min. Mass spectrometry was used to examine the gas chromatogram to determine the mass of each fraction. Retention was allowed for the identification of phytochemical components. The phytochemical compounds' identity was validated by comparing their peak area, retention time (RT), molecular formula, and retention index (RI) to the online NIST library chemistry web.

Component Identification. The molecular structure, molecular mass, and predicted fragments were used to identify the component. The GC/MS data are processed using the National Institute of Standards and Technology (NIST) database, comprising more than 62,000 patterns. It was possible to identify the components of the test sample by name, molecular weight, and structure. By comparison of the average peak of each component to the overall region, the relative proportion for each was determined. The unidentified component's spectrum was compared to the component's spectrum found in Turbos 5.2, NIST library version (2005).

Qualitative Analysis of Plant Extracts. We performed qualitative analysis of phytochemicals in methanol and aqueous extract of *R. hirtellus*, prepared by following the reported methods with some modifications.[33] Briefly, plant extracts (300 mg) were dissolved in DMSO (15 mL). The resulting extract solution (20 mg/mL) was titled "A/stock solution".

Test for Alkaloids. *Mayer's Test.* The Evan[34] method was used. Briefly, 500 μL of *R. hirtellus* (aerial part and roots) stock solutions were mixed with 1 mL of HCl, followed by two to three drops of Mayer's reagent. Red or yellowish-brown precipitates showed the presence of alkaloids.

Tannin Test. Kodangala and co-workers'[35] method was used. Briefly, one drop of ferric chloride (FeCl) was added to 500 μL of *R. hirtellus* stock solutions. A bluish-black color/greenish precipitate shows the existence of tannins.

Flavonoid Test. *Alkaline Reagent Test.* Xavier and Johnson's[36] method was used. In 500 μL of *R. hirtellus* stock solution, two drops of 5% NaOH were added. When 2−3 drops of HCl were added, an intensified yellow color appeared

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that eventually disappeared, showing the presence of flavonoids.

Test for Saponins. Singh and co-workers'[37] method was used. Briefly, stock solution (500 μL) was mixed vigorously with 2 mL of ultrapure water. After marking the froth's edges, it was watched for 10 min. Saponins are present when the foam remains.

Salkowski's Test for Sterols or Triterpenes. The procedure described previously[35] was used with changes. Briefly, stock solution (500 μL) was mixed thoroughly by vertexing with 1 mL of chloroform, and the chloroform's organic layer was isolated. Following separation, 0.5 mL of concentrated H_2SO_4 was supplemented with the chloroform extract, thoroughly mixed, and allowed to stand for 1 min. The presence of red color below the layer indicated sterols, whereas triterpenes were visible as indicated by yellow or golden yellow color on the top layer.

Test for Glycosides. *Sulfuric Acid Test.* Ahuja et al.,[38] method was used. Briefly, concentrated H_2SO_4 (1 mL) was added to 500 μL of *R. hirtellus* stock solutions. After vigorous shaking, the mixture was allowed to stand for 2 min. The presence of glycosides is indicated by the reddish-brown color's appearance.

Test for Amino Acid (Ninhydrin's Test). Ahuja and co-workers'[38] method was used. Briefly, ninhydrin solution (three drops) was added to 0.5 mL of stock solution and heated for 10 min in a water bath. The presence of amino acids was indicated by the emergence of a purple color.

Test for Carbohydrates (Benedict's Test). The method described by Kodangala and colleagues[35] was employed. Benedict's reagent was dissolved in 0.5 mL of *R. hirtellus* stock solutions, which was 0.5 mL in volume. For around 5 min, the mixture was heated in a boiling water bath. The indication of the reducing sugar was signaled by the appearance of a precipitate with a brick-red hue.

Anthocyanin Test. Savitharamma et al.,[39] method was used. A 200 μL *R. hirtellus* stock solution was mixed with 200 μL each of 2 N HCl and NH_3. Anthocyanins were shown by the blue-violet transformation of pink or red color.

Leucoanthocyanin Test. The method described by Savitharamma and colleagues[39] was used. 400 μL of *R. hirtellus* stock solutions were mixed with 0.4 mL of isoamyl alcohol. The presence of leucoanthocyanins was indicated by the appearance of a red color in the upper layer.

Test for Anthraquinones. Akinjogunla and co-workers'[40] method was used. Benzene (0.2 mL) and 0.4 mL of NH_3 were added to 200 μL of *R. hirtellus* stock solutions. The presence of anthraquinones was indicated by the emergence of either of three colors in the ammonical layer: pink, violet, or red.

Terpenoid Test. The method described by Singh and colleagues[37] was used. Chloroform (2 mL) and 0.5 mL of concentrated H_2SO_4 were added to 500 μL of *R. hirtellus* stock solutions. The presence of terpenoids was indicated by the reddish-brown color near the two-layer junction.

Test for Quinones. Rao and Savithramma's[41] methods were employed. Briefly, 0.5 mL of *R. hirtellus* stock solutions were mixed with 500 μL of alcoholic KOH. The presence of quinones was indicated by the emergence of color varying from red to blue.

Test for Lactones (Legal Test). The lactone test was performed using the method described by Ahuja and colleagues.[38] In 500 μL of pyridine, nearly 5 mg of the *R. hirtellus* stock solutions were dissolved. The aliquoted pyridine soluble extract was treated with a solution of sodium nitroprusside and NaOH. The presence of lactones was indicated by the dark red color.

Test for Lignin. *Labat Test.* Gibbs's[42] method as well as Rao and Savithramma's[41] method was employed. To this was added 0.5 mL of gallic acid (2.9 mg/mL) to 0.5 mL of *R. hirtellus* stock solutions. The olive-green color indicated the presence of lignin.

Dahlman Test. To check the presence of lignin, the method described by Haque and colleagues[43] was used. *R. hirtellus* stock solution (500 μL) was treated with aniline (two drops) and concentrated H_2SO_4 (a few drops). The presence of lignin is shown by the production of a yellow color.

Test for Phlobatannins. Pradeep and co-workers'[44] method was used with some modifications. *R. hirtellus* stock solution (500 μL) was added in 0.1 mL of HCl and then boiled for approximately 10 min. The formation of red precipitates was observed for phlobatannins.

QUANTITATIVE TESTS

Total Phenolic Test. Total phenolic contents were determined by the method reported by ref 45 by using a spectrophotometer with some modifications. Three concentrations of plant stock were taken, 125, 250, and 500 μg/mL. Methanol was employed as a control and gallic acid as a standard. Briefly, the *R. hirtellus* (aerial parts and roots separate) stock solutions of 5 g/mL were prepared. From the stock solutions, 30, 60, and 90 μL of plant extract from stock solutions were each mixed with 740 μL of distilled water. Following this, 60 μL of FC reagent was added, and the mixture was left to incubate for a duration of 5 min. After incubation, 500 μL of sodium carbonate was added and the mixture was incubated for 90 min. Following the incubation period, the absorbance was assessed by using a UV−vis spectrophotometer (Shimadzu UV-1800) at a wavelength of 550 nm. The positive control was gallic acid, and the control was methanol treated.

Total Flavonoid Test. The TFC was determined using the aluminum chloride colorimetric method which is reported by ref 46 with some modifications. The stock solution of 5 mg/mL *R. hirtellus* (aerial parts or roots) was prepared. From this stock, 25, 50, and 100 μL of plant extract were added to 300 μL of methanol, 20 μL of $AlCl_3$, 20 μL of potassium acetate, and 660 μL of distilled water one after another. The absorbance was measured after 30 min incubation, at 415 nm through a UV−vis spectrophotometer (Shimadzu UV-1800). Quercetin dehydrate was the positive control, and methanol was taken as a negative control.

Total Tannin Test. To determine the tannins, a Folin-Ciocalteu procedure was used which is reported by ref 47 with some modifications. 5 mg/mL of *R. hirtellus* plant (aerial parts or roots) was taken in a resultant solvent and prepared stock solution. From the stock solution, 25, 50, and 100 μL of plant extract were added to 50 μL of FC reagent. Then, 850 μL of distilled water and 100 μL of 35% Na_2CO_3 were added. Subsequently, 50 μL of distilled H_2O was added, and the mixture was allowed to incubate for a duration of 30 min. Following this incubation period, the absorbance was recorded at 725 nm by using a UV−vis spectrophotometer (Shimadzu UV-1800). Gallic acid served as the positive control, and methanol was utilized as the negative control.

Reducing Sugar Content (RSC). The assessment of the RSC was conducted using the 3,5-dinitrosalicylic acid (DNSA)

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method. The measurement was carried out using Krivorotova and Sereikaite's methodology[48] with slight modification. The DNSA reagent was made by dissolving 1 g of DNSA and 30 g of sodium−potassium tartaric acid in 80 mL of 0.5N NaOH at 45 °C. After dissolution, the mixture was cooled to ambient temperature and then diluted to a final volume of 100 mL with distilled water. A test tube containing 1 mL of plant extract (1 mg/mL) was pipetted with 2 mL of DNSA reagent, and the tube was stored at 95 °C for 5 min. The solution was allowed to cool before being mixed with 7 mL of distilled water. The absorbance of the solution was measured at 540 nm using a UV−vis spectrophotometer (Shimadzu UV-1800). The findings were expressed as milligrams of D-glucose equivalent (GE) per gram of dry extract weight. The reduced sugar content was determined using the standard D-glucose calibration curve (200−1000 mg/L).

Biological Activities. *Antidiabetic Test.* The antidiabetic effect of different extracts was assessed through an α-amylase inhibition assay.

α-Amylase. α-Amylase inhibitory activity of *R. hirtellus* was carried out using methanol and aqueous extracts according to the standard method reported by ref 49 with some modifications. Acarbose was used as a standard, and methanol was used as a negative control. *R. hirtellus* plant stock of (aerial parts or roots) 5 mg/mL in DMSO was taken as a stock solution, and three concentrations were taken from plant stock, i.e., 125, 250, and 500 μL/mL. Further, 160 μL of α-amylase and 60 μL of phosphate buffer solution were added, followed by incubation for 20 min at 37 °C. Subsequently, 80 μL of starch solution was added and incubated again in boiling water at 37 °C for 15 min. Next, 400 μL of DNS was added and diluted with a dilution factor, i.e., distilled water. Acarbose was used as the standard. Absorbance was measured at 540 nm using a UV−vis spectrophotometer (Shimadzu UV-1800). The formula was used to calculate the percentage inhibition:

$$\%\ \text{inhibition} = \frac{\text{control} - \text{sample}}{\text{control}} \times 100$$

The inhibitory property represented by the sample was compared to the control and then expressed as percentage inhibition.

Antioxidant. The antioxidant effect of different extracts was assessed through DPPH.

Diphenyl—Picryl Hydroxyl (DPPH). The DPPH assay was performed according to the Mruthunjaya and Hukkeri method with some changes.[50] Briefly, 5 mg/mL of *R. hirtellus* stock solution (aerial parts or roots) was prepared in methanol. Three concentrations (125, 250, 500 μg/mL) with 12.5, 25, and 50 μL were taken. All of these concentrations were then raised to 1 mL with methanol. Out of these, 100 μL with three replications was taken in 96-well plates, and 100 μL of DPPH methanol solution was added. The samples were incubated for 30 min, and the absorbance was taken at 517 nm on a UV−vis spectrophotometer (Shimadzu UV-1800). Ascorbic acid was used as the standard, and methanol was used as the control. The following formula was used to calculate the percentage inhibition.

$$\%\ \text{inhibition} = \frac{\text{control} - \text{sample}}{\text{control}} \times 100$$

ABTS Radical Scavenging Assay. The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation (ABTS•+)] method[51] was used to evaluate the free-radical-scavenging effect of the samples with slight modification. A total of 5 mg of ABTS (Glentham Life Sciences, Corsham, U.K.) was dissolved in 7 mL of phosphate-buffered saline (PBS), (Sigma-Aldrich, St. Louis, MO) resulting in a final ABTS concentration of 0.7 mM. Simultaneously, a 2.45 mM solution of potassium persulfate (VWR Chemicals BDH Prolabo, Australia) was prepared in deionized water. Subsequently, 7.0 mL of the ABTS solution was combined with 7.0 mL of the potassium persulfate solution in a glass tube. This mixture was then placed in the dark at room temperature (24 ± 1 °C). Antioxidant assessment was conducted immediately after the oxidation of ABTS, ensuring complete formation of the ABTS radical. Different concentrations of the test samples were prepared in DMSO, and an aliquot of 50 μL was transferred to a 96-well plate. To this, 150 μL of the ABTS reaction mixture was added to each well and incubated at room temperature for 10 min. After incubation, the absorbance was measured at 734 nm using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT). A blank was also applied having DMSO and ABTS solution. The ABTS scavenging effect was measured using the following formula:

$$\text{radical scavenging}\ (\%) = \frac{A_{\text{control}} - B_{\text{sample}}}{A_{\text{control}}} \times 100$$

where *A*-control is the absorbance of blank and *B* is the absorbance of samples.

STATISTICAL ANALYSIS

The statistical data were analyzed through Graph Prism ver. 6.01 software and SPSS version 16. One-way analysis of variance (ANOVA) was performed through SPSS to determine the significant difference ($p < 0.05$) between the samples.

RESULTS

Phytochemical Detection through GCMS. The gas chromatography−mass spectrometry (GCMS) analysis revealed the presence of various phytochemical compounds in the aerial parts and roots of *R. hirtellus*, extracted using ethyl acetate, chloroform, and *n*-hexane solvents. *R. hirtellus* reveals the presence of thirty-three compounds overall. In the ethyl acetate extract of the aerial parts, four identified compounds are neophytadiene at 6.266 min (30.72%), succinic acid, tridec-2-yn-1-yl 4-octyl ester at 2.424 min (8.41%), hexacosyl acetate2,6,10,14- at 5.936 min (5.0009%), and phytyl tetradecanoate at 6.496 min (0.54671%). Conversely, in the ethyl acetate extract of the roots, six compounds, i.e., 2-oxepanone, 7-hexyl- at 3.48 min (54.644%), succinic acid, but-3-Yn-2-Yl *cis*-4-methylcyclohexyl ester at 2.424 min (12.62%), hentriacontane at 2.83 min (1.62%), mesitylene at 2.374 min (1.045%), dotriacontyl pentafluoropropionate at 4.465 min (0.17%), and malonic acid, 2-butyl heptadecyl ester at 2.519 min (0.09%), were detected.

Chloroform extracts exhibited six compounds, i.e., methanoazulene, octahydro-1,4,9,9- at 2.419 min (35.29%), tetramethyl-11,14-phenol, 2,5-bis(1,1-dimethylethyl)- at 4.805 min (34.18%), neophytadiene at 6.206 min (10.013%), glutaric acid, tridec-2-yn-1-yl 3, 7-dimethyloctyl ester" at 4.69 min (1.15%), octadecane, 1-chloro- at 4.29 min (0.63%), and 11,14-eicosadienoic acid, methyl ester at 5.596 min (0.29%), in aerial parts, while 2(3h)-furanone, 5 dodecyldihydro- at 3.365 min (60.01%), hentriacontane at 5.601 min (26.27%), 5-fluoro-2-trifluoromethylbenzoic acid, 5-pentadecyl ester at

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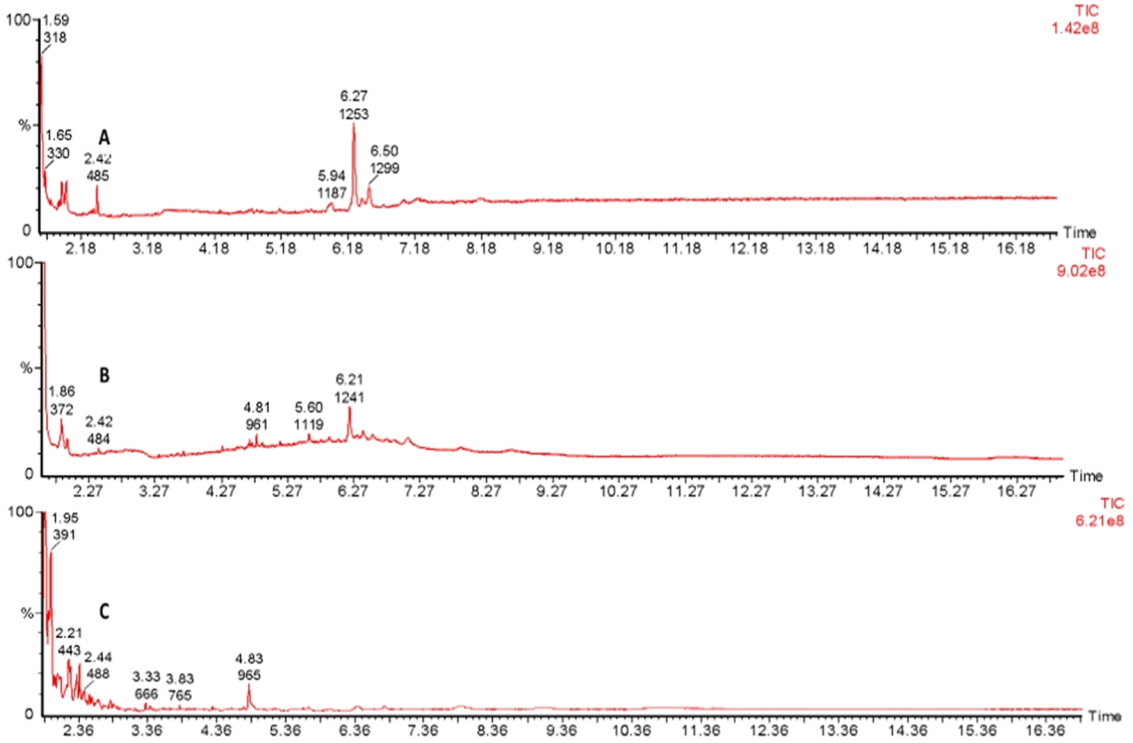

Figure 1. *R. hirtellus* aerial parts' GC/MS graph representing peaks and nodes of different compounds, i.e., (A) peaks of ethyl acetate, (B) peaks of chloroform, and (C) peaks of *n*-hexane.

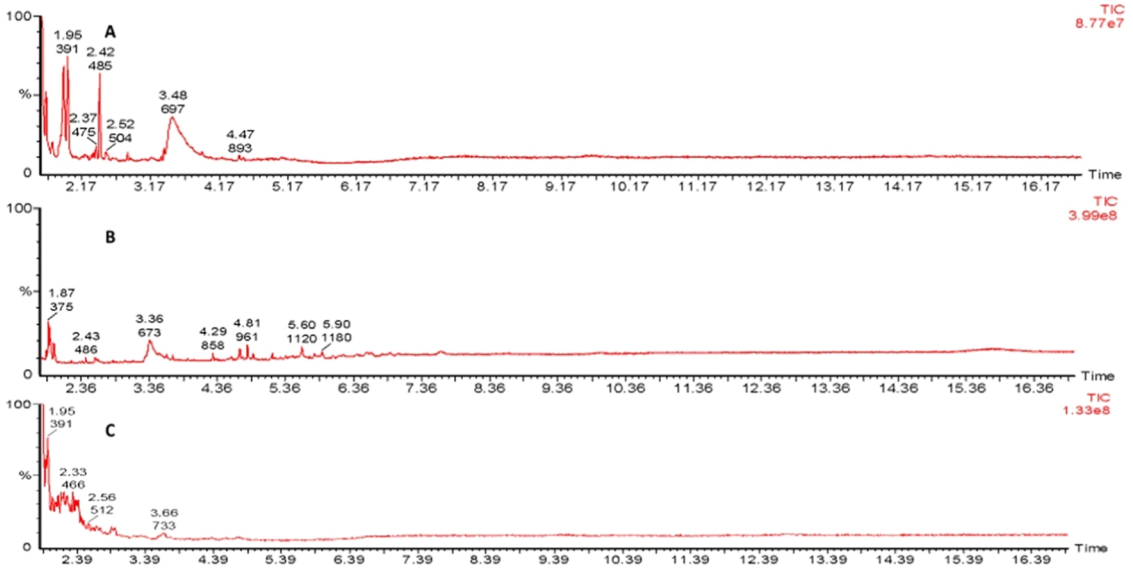

Figure 2. *R. hirtellus* roots' GC/MS graph representing peaks and nodes of different compounds, i.e., (A) peaks of ethyl acetate; (B) peaks of chloroform; and (C) peaks of *n*-hexane.

4.805 min (3.56%), carbonic acid, octadecyl vinyl ester at 4.29 min (2.79%), dotriacontyl pentafluoropropionate at 5.90 min (2.03%), and succinic acid, tridec-2-yn-1-yl 2-ethylbutyl ester at 2.429 min (0.19%) are detected in the root chloroform extract.

Notable six compounds in *n*-hexane extracts are detected including 2,4-di-*tert*-butylphenol at 4.825 min (16.71%), benzene, 1-ethyl-3-methyl- at 2.214 min (9.51%), benzene, 1,2,4-trimethyl- at 2.374 min (7.43%), hentriacontane at 2.822 min (2.73%), eicosyl nonyl ether at 3.33 min (0.94%), and hentriacontane at 3.825 min (0.27%), in aerial parts, while in the root *n*-hexane extract, five compounds, i.e., chloroacetic acid, tetradecyl ester at 2.114 min (22.22%), nonyl octacosyl ether at 3.66 min (12.79%), phytyl, 2-methylbutanoate at 2.89 min (10.51%), malonic acid, 2-butyl heptadecyl ester at 2.564 min (3.25%), and tumerone at 2.404 min (2.51%), are detected. A total of 14 different compounds were identified in each part, i.e., aerial and roots. These findings underscore the diverse phytochemical composition of *R. hirtellus*, potentially contributing to its therapeutic properties. Chromatograms depicting compound peaks are provided in Figures 1 and 2, with detailed compound information in Tables 1 and 2.

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Additionally, the chemical structures of these compounds are depicted in Figure 3.

Qualitative Analysis of Phytochemicals in the Plant Crude Extract. The phytochemicals were identified in two different extracts of methanol and aqueous. The results revealed that 16 compounds have been identified as presented in Table 3. In the *R. hirtellus* aerial parts, flavonoids, sterols, glycosides, quinones, lactones, and lignin were present in the methanolic extract. Additionally, alkaloids sterols, glycoside, quinones, and lignin (Dahlman test) were present in aqueous extracts. Carbohydrates, tannins, saponins, amino acids, anthocyanins, anthraquinone, and phlobatannins were absent in both methanolic and aqueous extracts. Alkaloids, leucoanthocyanins, terpenoids, and triterpenes were present in aqueous extracts but absent in the methanolic extract, while flavonoids and lignin were present in methanolic extracts but absent in the aqueous extract. In *R. hirtellus* roots, the flavonoids, sterol, glycosides, quinones, and lignin were present in methanolic extracts. In the same extracts, alkaloids, tannins, saponins, amino acids, carbohydrates, anthocyanins, leucoanthocyanins anthraquinones, phlobatannins, triterpene, lactones, and terpenoids were absent.

In *R. hirtellus* roots, aqueous extract terpenoids, quinones, alkaloids, sterols, triterpene, glycosides, carbohydrates, leucoanthocyanins, and lactones were present. Additionally, in aqueous extracts, phlobatannins, anthraquinones, lignin, anthocyanins, amino acids, saponins, flavonoids, and tannins were absent. Quinones, glycosides, and sterols were present in both extracts of methanol and aqueous, while in both extracts (methanol and aqueous), amino acid, saponins, tannins, anthocyanins, anthraquinones, lignin (labat test), and phlobatannins were absent. These results are summarized in Table 3.

Quantitative Analysis of Total Tannin, Phenolic, Flavonoid Content, and Carbohydrates in Plant Crude Extracts. The TTC in both methanolic and aqueous extracts of *R. hirtellus* is expressed as gallic acid equivalent (mg of GAE/g extract). The aqueous extract (aerial parts and roots) showed a strong linear relationship between TTC and plant concentration ($y = 6 \times 10^{-07}x + 0.4996$, $R^2 = 0.9992$) and ($y = 8 \times 10^{-08}x + 0.3786$, $R^2 = 0.9992$), and methanol extracts of both parts showed a weaker relationship ($y = 3 \times 10^{-07}x + 0.4998$, $R^2 = 0.4902$) and ($y = 1 \times 10^{-07}x + 0.3785$, $R^2 = 0.693$) respectively. The standard TTC for the aerial parts of *R. hirtellus* was determined using gallic acid (2 mg/mL) and showed a strong linear relationship ($y = 0.0006x + 0.3783$, $R^2 = 0.9996$). The TTC values were 0.451333, 0.53, and 0.676 μg/mL for gallic acid concentrations of 125, 250, and 500 μg/mL, respectively. In comparison, the TTC values for both the aqueous and methanol extracts of *R. hirtellus* (aerial parts and roots) were approximately 0.3786 μg/mL across all plant concentrations. However, the TTC values for the methanol extracts showed a slight increase with increasing plant concentration, ranging from approximately 0.3785 to 0.3786 μg/mL. These results are further summarized in Figure 4A–E and Table 4.

The study investigated the total phenolic content (TPC) in both methanolic and aqueous extracts of *R. hirtellus*, expressed as milligrams of gallic acid equivalent (GAE) per gram of extract. TPC in the aqueous (ariel) extract showed a strong linear relationship ($y = 1 \times 10^{-07}x + 0.0476$, $R^2 = 0.9024$), while the methanol extract showed a weaker relationship ($y = 2 \times 10^{-07}x + 0.0476$, $R^2 = 0.8535$). TPC in the aqueous (roots)

Table 1. Compounds Present in *R. hirtellus* Aerial Parts Detected through GC/MS

plant	extract	retention time	class	name of compound	antioxidant/antidiabetic	area (%)	retention index	MW	formula
R. hirtellus aerial part	ethyl acetate	2.424	ester	succinic acid, tridec-2-yn-1-yl 4-octyl ester		8.40993	2771	408	$C_{25}H_{44}O_4$
		5.936	acetate ester	hexacosyl acetate2,6,10,14-	antidiabetic[52]	5.00885	2995.6	424	$C_{28}H_{56}O_2$
		6.266	terpene	neophytadiene	antioxidant[53]	30.7171	1836	278	$C_{20}H_{38}$
		6.496	ester	phytyl tetradecanoate		0.54671	3361.9	506	$C_{34}H_{66}O_2$
	chloroform	2.419	sesquiterpene	octahydro-1,4,9,9-tetramethyl-1H-3a,7-methanoazulene		35.288	2.419	206	$C_{15}H_{26}$
		4.29	haloalkane	octadecane, 1-chloro-		0.62651	346.7	288.9	$C_{18}H_7Cl$
		4.69	ester	glutaric acid, tridec-2-yn-1-yl 3, 7-dimethyloctyl ester"		1.15099	3080	450.6	$C_{28}H_{50}O_4$
		4.805	phenol derivative	tetramethyl-11,14-phenol, 2,5-bis(1,1-dimethylethyl)-		34.1775	1517	206	$C_{14}H_2O$
		5.596	unsaturated fatty acid ester	11,14-eicosadienoic acid, methyl ester		0.28902	2285	404	$C_{29}H_{40}O$
		6.206	terpene	neophytadiene	antioxidant[53]	10.0133	1836	278	$C_{20}H_{38}$
	n-hexane	2.214	aromatic hydrocarbon	benzene, 1-ethyl-3-methyl-		9.5145	963.9	120	C_9H_{12}
		2.374	aromatic hydrocarbon	benzene, 1,2,4-trimethyl-	antioxidant[54]	7.4268	976	120	C9H12
		2.822	alkane	hentriacontane		2.7338	478.6	436	$C_{31}H_{64}$
		3.33	ether	eicosyl nonyl ether		0.9417	2957.	424	$C_{29}H60O$
		3.825	alkane	hentriacontane		0.2701	478.6	436	$C_{31}H_{64}$
		4.825	phenol derivative	2,4-di-*tert*-butylphenol	antioxidant[55]	16.714	1512	206	$C_{14}H_{22}O$

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Table 2. Compounds Present in *R. hirtellus* Roots Detected through GC/MS

plant	extract	RT	class	name of compound	antioxidant/antidiabetic	area (%)	RI	MW	formula
R. hirtellus roots	ethyl acetate	2.374	aromatic hydrocarbon	mesitylene		1.064858	995	120	C_9H_{12}
		2.424	carboxylic acid esters	succinic acid, but-3-Yn-2-Yl *cis*-4-methylcyclohexyl ester		12.62684	1798	266	$C_{15}H_{22}O_4$
		2.519	carboxylic acid esters	malonic acid, 2-butyl heptadecyl ester		0.086422	2667	398	$C_{24}H_{46}O_4$
		2.83	alkanes	'hentriacontane		1.620234	478.7	436	$C_{31}H_{64}$
		3.48	lactones	2-oxepanone, 7-hexyl-		54.644	2264	198	$C_{12}H_{22}O_2$
		4.465	carboxylic acids	dotriacontyl pentafluoropropionate		0.169998	3351.4	612	$C_{35}H_{65}F_5O_2$
	chloroform	2.429	carboxylic acid alkynes	succinic acid, tridec-2-Yn-1-Yl 2-ethylbutyl ester		0.190708	2683	380	$C_{23}H_{40}O_4$
		3.365	ketones	2(3h)-furanone, 5 dodecyldihydro-		60.01226	2106	254	$C_{16}H_{30}O_2$
		4.29	carboxylic acid ester	carbonic acid, octadecyl vinyl ester		2.796331	2299	340	$C_{21}H_{40}O_3$
		4.805	carboxylic acid esters	5-fluoro-2-trifluoromethylbenzoic acid, 5-pentadecyl ester		3.561565	2241	418	$C_{23}H_{34}F_4O_2$
		5.601	alkanes	hentriacontane		26.26843	478.7	436	$C_{31}H_{64}$
		5.90	carboxylic acids	dotriacontyl pentafluoropropionate		2.026320	3351.4	612	$C_{35}H_{65}O_2F_5$
	n-hexane	2.329	aromatic hydrocarbon	mesitylene		22.21987	996.4	120	C_9H_{12}
		2.404	sesquiterpenes	turmerone	antioxidant and antidiabetic[56]	2.508004	1654	218	$C_{13}H_{22}O$
		2.564	carboxylic acid esters	malonic acid, 2-butyl heptadecyl ester		3.253611	2667	398	$C_{24}H_{46}O_4$
		2.89	carboxylic acid esters	phytyl, 2-methylbutanoate		10.51209	2440.9	380	$C_{25}H_{48}O_2$
		3.66	ethers	nonyl octacosyl ether		12.78851	3747	536	$C_{37}H_{76}O$

extract showed a strong linear relationship ($y = 1 \times 10^{-07}x + 0.0476$, $R^2 = 0.8462$), while the methanol extract showed a stronger relationship ($y = 2 \times 10^{-07}x + 0.0476$, $R^2 = 0.9209$). The standard TPC for the aerial parts of *R. hirtellus* was determined using gallic acid (2 mg/mL) and showed a strong linear relationship ($y = 0.0007x + 0.0475$, $R^2 = 0.9697$). The TPC values were 0.116333, 0.247, and 0.384667 μg/mL for gallic acid concentrations of 125, 250, and 500 μg/mL, respectively. In comparison, the TPC values for both the aqueous and methanol extracts of *R. hirtellus* (aerial parts and roots) were approximately 0.0476 μg/mL across all plant concentrations. These results are further summarized in Figure 5A−E and Table 4.

The study investigated the total flavonoid content (TFC) in both methanol and aqueous extracts of *R. hirtellus*, expressed as milligrams of quercetin equivalent (QE) per gram of extract. In both (aerial parts and roots) of *R. hirtellus*, the TFC in the aqueous extract showed a strong linear relationship ($y = 1 \times 10^{-06}x + 0.1864$, $R^2 = 0.9909$) and ($y = 1 \times 10^{-06}x + 0.1862$, $R^2 = 0.9976$), while the methanolic extract showed a weaker relationship ($y = 3 \times 10^{-06}x + 0.1864$, $R^2 = 0.6542$) and ($y = 3E07x + 0.1863$, $R^2 = 0.9481$), respectively. The standard TFC for the aerial parts of *R. hirtellus* was determined using quercetin dehydrate and showed a strong linear relationship ($y = 0.0023x + 0.1861$, $R^2 = 0.9463$). The TFC values increased from 0.3423 at 100 μg/mL to 1.2903 at 500 μg/mL in standard quercetin. In comparison, the TFC values for both the aqueous and methanol extracts of *R. hirtellus* (aerial parts and roots) were lower than the standard values but showed a slight increase with the plant concentration. These results are summarized in Figure 6A−E and Table 4.

The reducing sugar content (RSC) in four different extracts (chloroform, ethanol, *n*-hexane, and methanol) of *R. hirtellus* aerial parts expressed in terms of D-glucose equivalent (mg of GE/g of extract). In chloroform, RSC values ranged from 0.9348 to 0.9475 μg/mL, with an *R*-value of 0.968762097. Ethanol exhibited RSC values between 0.9367 and 0.9583 μg/mL, with an *R*-value of 0.975704873. Similarly, *n*-hexane showed RSC values from 0.9336 to 0.9555 μg/mL, with an *R*-value of 0.988787136. Methanol displayed RSC values ranging from 0.9379 to 1.0119 μg/mL, with an *R*-value of 0.988787136. These results are depicted in Figure 7A−D and Table 5.

Effect of Plant Crude Extracts on α-Amylase (In Vitro). α-Amylase inhibitory tests were used to test antidiabetic efficacy in the methanolic, aqueous extracts of *R. hirtellus* aerial parts (except the flower). α-Amylase inhibition was measured by using acarbose as the standard. In addition, the highest percentage of amylase inhibition was recorded in both methanolic and aqueous extracts at a dose of 500 μg/mL, while the lowest concentration was (125 μg/mL). The α-amylase inhibitory experiment was performed on methanolic and aqueous extracts at different doses (125, 250, and 500 μg/mL). *R. hirtellus* aerial part methanolic extracts showed inhibition at concentrations of 125 μg/mL (65.47 ± 1.67%), 250 μg/mL (67.17 ± 1.95%), and 500 μg/mL (68.37 ± 2.42%). Their aqueous extracts exhibited inhibition at concentrations of 125 μg/mL (60.65 ± 2.4217%), 250 μg/mL (65.97 ± 0.5917%), and 500 μg/mL (70.12 ± 1.025%). The highest percentage inhibition, reaching 70.12 ± 1.025%, was observed with the aqueous extract at a concentration of 500 μg/mL. Conversely, the lowest inhibition percentage, at

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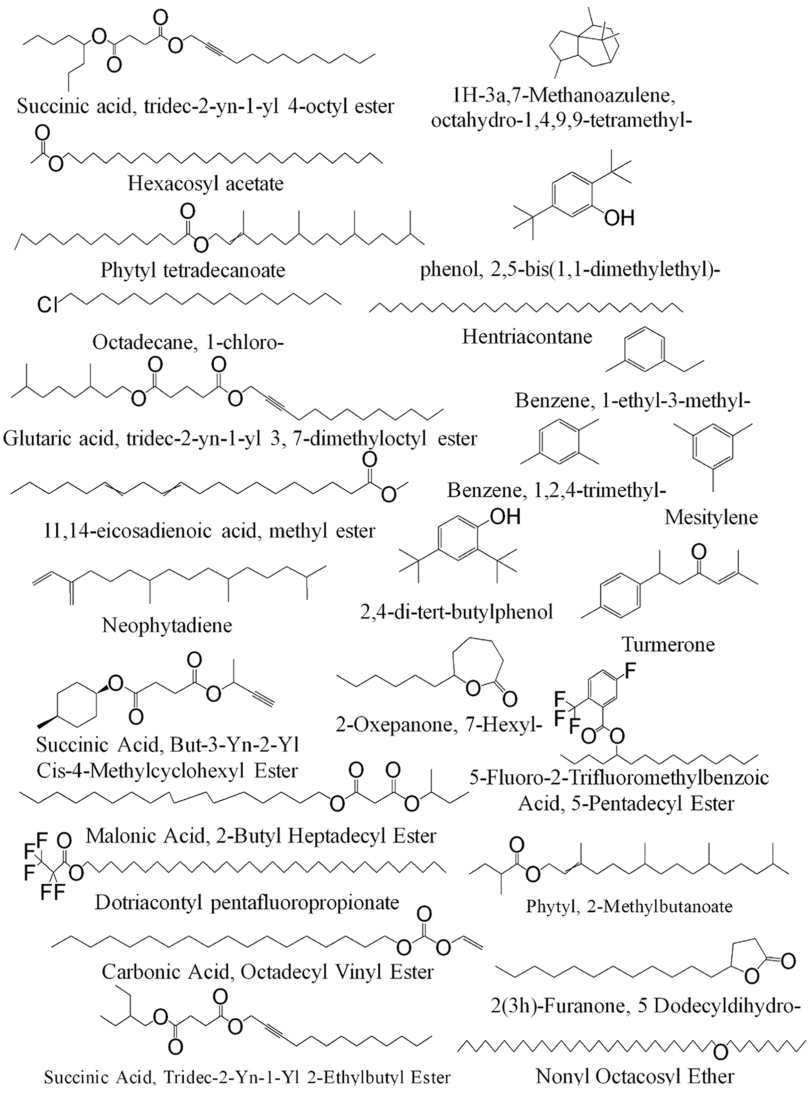

Figure 3. Chemical structure compounds detected in different extracts of *R. hirtellus* leaves and roots. ChemDraw software was used to draw the original structures.

60.65 ± 2.4217%, was noted with the same extract but at a concentration of 125 μg/mL.

In methanolic extract of *R. hirtellus* roots, the inhibition is at concentrations of 125 μg/mL (51.77515 ± 1.47929%), 250 μg/mL (68.63905 ± 0.51244%), and 500 μg/mL (71.3018 ± 1.28962%), while the inhibition is at concentrations of 125 μg/mL (48.4221 ± 3.25892%), 250 μg/mL (63.70818 ± 2.1808%), and 500 μg/mL (69.3294 ± 0.45193%) in the aqueous extract. The highest percentage inhibition at a concentration of 500 μg/mL (71.3018 ± 1.28962%) was achieved in the methanolic extract, while the lowest percentage inhibition at a concentration of 125 μg/mL (48.4221 ± 3.25892%) was achieved in the aqueous extract. The IC_{50} value was calculated online through the IC_{50} calculator AAT Bioquest. The IC_{50} values of *R. hirtellus* aerial parts for acarbose and the extract of methanol were determined to be 270. and 241.39, respectively, while the IC_{50} value of aqueous was 240.651. The IC_{50} values of *R. hirtellus* roots for acarbose and the root extract of methanol were determined to be 270.0029 and 207.8264 μg/mL, respectively. The IC_{50} value for the root extract of aqueous was 225.8938 μg/mL. The percentage inhibition and IC_{50} value displayed by each extract are shown in Table 6. A comparison of the percentage inhibition of the amylase−amylase enzyme with acarbose and *R. hirtellus* at different concentrations is presented in Figure 8. The corresponding *p*-values can be found in the Supporting File (Figure 11S). The graph of *R. hirtellus* aerial parts shows that in the methanolic extract, all three concentrations are significantly different ($p < 0.05$) and dose-dependently increase compared to the standard. In comparison, the three concentrations show dose-dependent trends but are not significant. However, in the aqueous extract, all concentrations exhibit significant differences ($p < 0.05$) and dose-dependently increase compared to the standard. Specifically, concentrations of 250 and 500 show a dose-dependent increase and are significant compared to 125, but 500 is nonsignificant compared to 250. The *R. hirtellus* root graph shows that in both extracts, all three concentrations are significantly different ($p < 0.05$) and dose-dependently increased compared to the standard. In methanol, 250 was nonsignificant and dose-dependently increased compared to 500, while in the aqueous extract, all concentrations were significantly different ($p < 0.05$) and dose-dependently increased compared to each other.

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Table 3. Phytochemical Screening of *R. hirtellus* Aerial and Root Part of Methanolic and Aqueous Extract Presence: + , Absence: −

test		resultant colors	methanol		aqueous	
			R.H-A.P	R.H-RT	R.H-A.P	R.H-RT
alkaloids		yellow brown/white precipitate	−	−	+	+
tannins		bluish-black color	−	−	−	−
flavonoids (alkaline reagent test)		yellow color faded away after HCl addition	+	+	−	−
saponins		froth appearance	−	−	−	−
Salkowski's test	sterols	red color in the lower layer	+	+	+	+
	triterpenes	yellow or golden color	−	−	+	+
glycosides (sulfuric acid test)		reddish-brown color	+	+	+	+
amino acid (Ninhydrin test)		purple color	−	−	−	−
carbohydrates (Benedict's test)		brick-red precipitates	−	−	−	+
anthocyanins		pink-turns-violet	−	−	−	−
leucoanthocyanins		red color in the upper layer	−	−	+	+
anthraquinones		pink/red/violet	−	−	−	−
terpenoids		reddish-brown color	−	−	+	+
quinone's		red to blue color	+	+	+	+
legal test (lactones)		deep red color	+	−	+	+
lignin	labat test	olive-green color formation	+	−	−	−
	Dahlman test	yellow color formation	−	+	−	−
phlobatannins		red precipitates	−	−	−	−

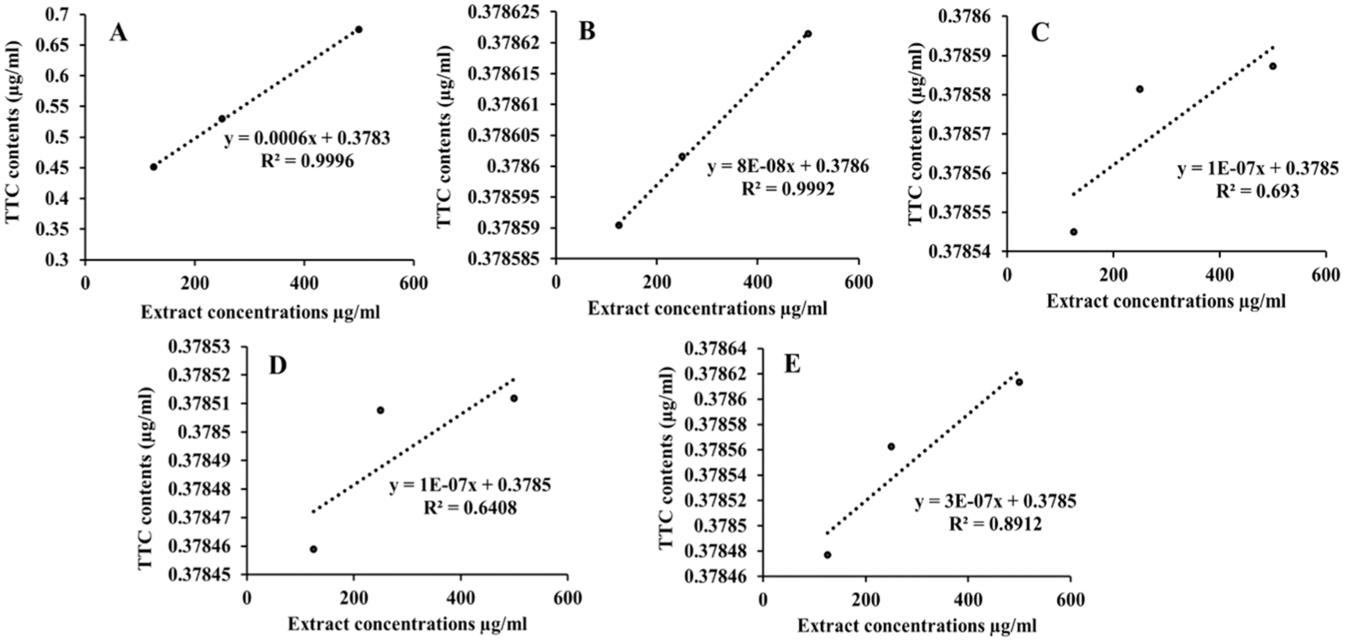

Figure 4. Correlations between TTC and concentrations of *R. hirtellus* extracts. (A) Standard, (B) *R. hirtellus* aerial part aqueous, (C) *R. hirtellus* aerial part methanol, (D) *R. hirtellus* root aqueous, and (E) *R. hirtellus* root methanol.

Effect of Plant Crude Extracts on DPPH (In Vitro). Radical scavenging activities of methanolic extracts of plants were checked through DPPH. The results revealed that both the extracts of *R. hirtellus* inhibited DPPH free radicals dose-dependently, except for the *R. hirtellus* root aqueous extract which was nonsignificant and neither increased dose-dependently. The DPPH free-radical inhibitory experiment was performed on methanol and aqueous extracts at different concentrations (125, 250, 500 µg/mL). In *R. hirtellus* aerial parts, the inhibition was at concentrations of 125 µg/mL (73.1 ± 0.18%), 250 µg/mL (75.9 ± 1.65%), and 500 µg/mL (82.13 ± 0.58%) in the methanolic extract, while in aqueous, it was at 125 µg/mL (67.87 ± 2.0%), 250 µg/mL (71.60 ± 1.452%), and 500 µg/mL (74.02 ± 0.462%). In *R. hirtellus* aerial parts, the highest percentage of DPPH inhibition was recorded in the methanolic extract at a dose of 500 µg/mL (82.13 ± 0.58%) and in the aqueous extract at a dose of 500 µg/mL (74.02 ± 0.46%), while the lowest was at 125 µg/mL in both methanolic and aqueous extracts, as represented in Figure 9. The IC_{50} values for ascorbic acid, methanolic, and aqueous extract of *R. hirtellus* aerial parts were determined to be 70.71, 270.28, and 239.28 µg/mL, respectively. Figure 9 shows a significant relation and dose-dependent increase in methanol. In aqueous, 250 and 500 were dose-dependently and significantly increased ($p < 0.05$) compared to 125, while 500 was dose-dependently increased but not significantly as compared to 250.

In *R. hirtellus* roots, the inhibition was observed at concentrations of 125 µg/mL (73.4383 ± 0.52295%), 250

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Table 4. Quantitative Test Results According to Three Different Concentrations of the *R. hirtellus* Aqueous Extract and Roots[a]

		quantitative test results					
s.no	plants	conc. plant	conc (μg/mL)	TTC-gallic acid (μg/mL)	TPC-gallic acid (μg/mL)	conc. (μg/mL)	TFC-quercetin (μg/mL)
1.	standard	125	125	0.451333	0.116333	100	0.3423
		250	250	0.53	0.247	200	0.6633
		500	500	0.676	0.384667	300	1.025
						400	1.083
						500	1.2903
	R-value			0.99979998	0.984733466	0.972779523	
				aqueous			
2.	*R. hirtellus* (aerial)	125	125	0.378590	0.0476	0.186587	
		250	250	0.378602	0.0476	0.186714	
		500	500	0.378621	0.0476	0.187100	
	R-value			0.99959992	0.949947367	0.995439601	
	R. hirtellus (root)	125	125	0.378459	0.047576	0.18643	
		250	250	0.378508	0.04761	0.1866	
		500	500	0.378512	0.047628	0.18698	
	R-value			0.800499844	0.919891298	0.998799279	
				methanol			
3.	*R. hirtellus* (aerial)	125	125	0.378545	0.0476	0.186424	
		250	250	0.378581	0.0476	0.187445	
		500	500	0.378587	0.0476	0.187550	
	R-value			0.832466216	0.923850637	0.808831	
	R. hirtellus (root)	125	125	0.378477	0.047568	0.18637	
		250	250	0.378563	0.047605	0.18638	
		500	500	0.378613	0.047632	0.18648	
	R-value			0.944033898	0.959635347	0.973704267	

[a]Conc. means concentration.

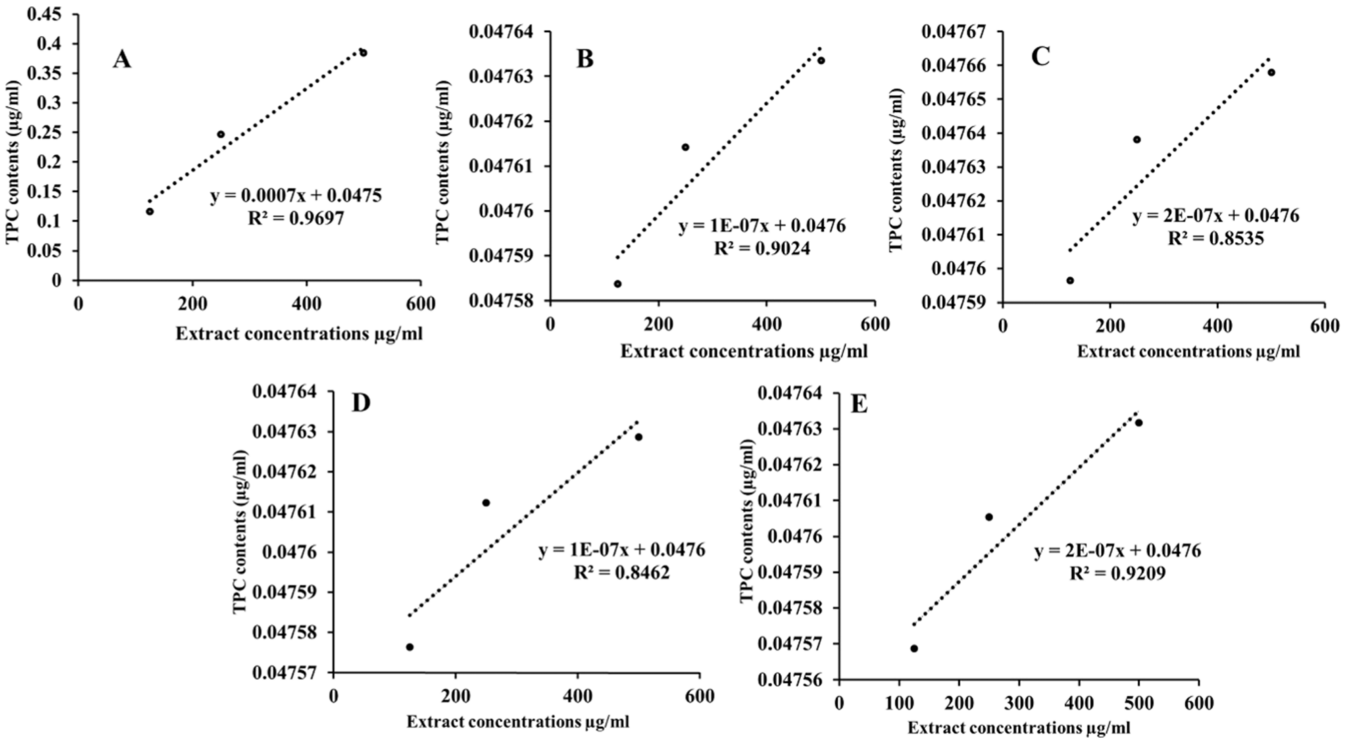

Figure 5. Correlations between TPC and concentrations of the *R. hirtellus* extracts. TPC (A) Standard, (B) *R. hirtellus* aerial part aqueous, (C) *R. hirtellus* aerial part methanol, (D) *R. hirtellus* root aqueous, and (E) *R. hirtellus* root methanol.

μg/mL (75.5077 ± 1.10564%), and 500 μg/mL (77.7948 ± 0.35986%) in the methanolic extract, while in aqueous, it was at 125 μg/mL (75.0128 ± 0.53227%), 250 μg/mL (74.9871 ± 0.49593%), and 500 μg/mL (74.6117 ± 3.829485%). The highest DPPH inhibition percentage, 75.0128 ± 0.53227%, was observed in the aqueous extract at a dose of 125 μg/mL. Conversely, the lowest inhibition percentage, 74.611 ± 3.829485%, was noted in *R. hirtellus* roots at a concentration

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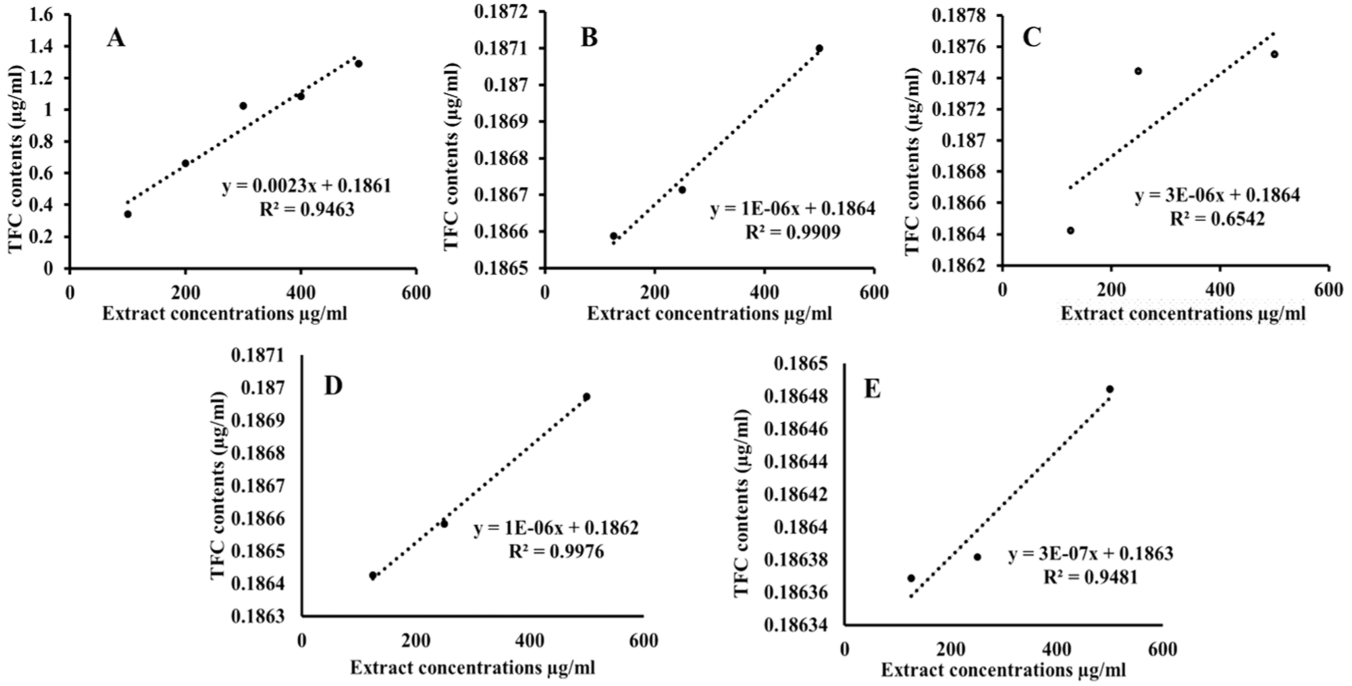

Figure 6. Correlations between TFC and concentrations of *R. hirtellus* extracts. TFC (A) Standard, (B) *R. hirtellus* aerial part aqueous, (C) *R. hirtellus* aerial part methanol, (D) *R. hirtellus* root aqueous, and (E) *R. hirtellus* root methanol.

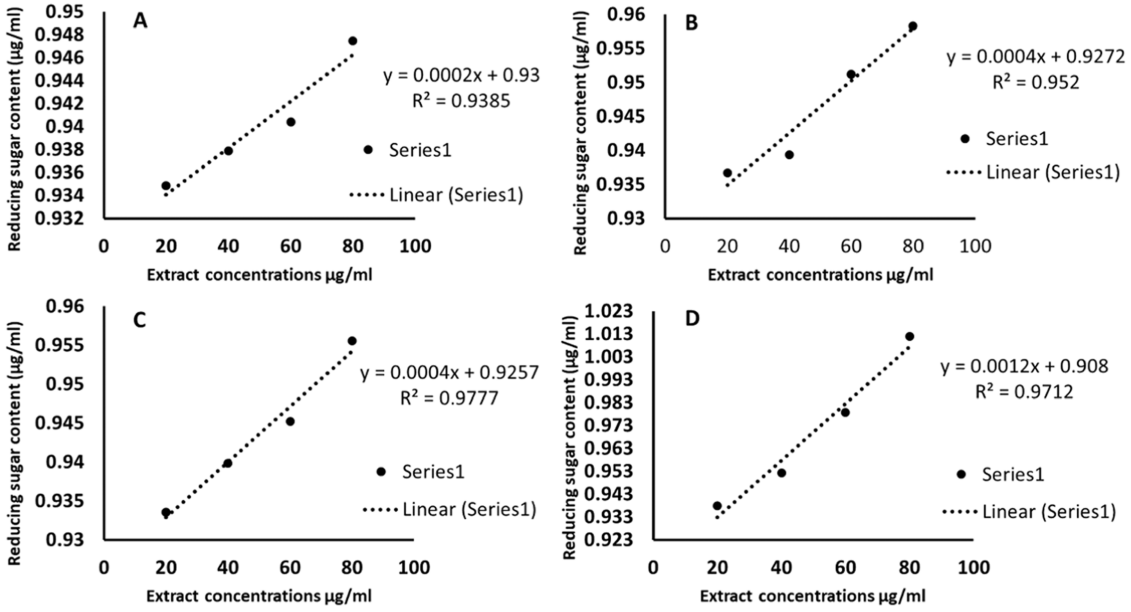

Figure 7. Correlations between RSC and concentrations of *R. hirtellus* aerial part extracts. (A) Chloroform, (B) ethanol, (C) *n*-hexane, (D) methanol.

Table 5. Quantitative Test for RSC According to Four Different Concentrations of the *R. hirtellus* Aerial Part

conc. (µg/mL)	RSC-chloroform (µg/mL)	RSC-ethanol (µg/mL)	RSC-*n*-hexane (µg/mL)	RSC-methanol (µg/mL)
20	0.9348	0.9367	0.9336	0.9379
40	0.9379	0.9393	0.9399	0.9522
60	0.9404	0.9512	0.9452	0.9785
80	0.9475	0.9583	0.9555	1.0119
R-value	0.968762097	0.975704873	0.988787136	0.988787136

of 500 µg/mL. The IC50 values for ascorbic acid, methanol, and aqueous extracts of *R. hirtellus* roots were determined to be 43.34, 252.52, and 325.33 µg/mL, respectively, as shown in Figure 9. Corresponding *p*-values can be found in the Supporting File (Figure S1). The graph showed a significant relation and a dose-dependent increase in methanol. In

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Table 6. Percentage Inhibition of α-Amylase, DPPH, and ABTS Activity with Their IC_{50} Values

% inhibition/concentration of biological activities					
	test		concentration	% inhibition	IC_{50} value
α-amylase	*R. Hirtellus* aerial parts	methanol	125 μg/mL	65.97633 ± 1.647	241.399 μg/mL
			500 μg/mL	68.83629 ± 2.391	
		aqueous	125 μg/mL	60.65089 ± 2.422	240.651 μg/mL
			500 μg/mL	70.11834 ± 1.025	
	R. Hirtellus roots	methanol	125 μg/mL	51.77515 ± 1.4793	207.8264 μg/mL
			500 μg/mL	71.3018 ± 1.2896	
		aqueous	125 μg/mL	48.4221 ± 3.259	225.8938 μg/mL
			500 μg/mL	69.3294 ± 0.452	
	acarbose (standard)		500 μg/mL	74.65483 ± 0.005	270.006 μg/mL
DPPH	*R. Hirtellus* aerial parts	methanol	125 μg/mL	73.0812 ± 0.1838	270.4837 μg/mL
			500 μg/mL	82.129 ± 0.58134	
		aqueous	125 μg/mL	67.86792 ± 2.0001	239.2857 μg/mL
			500 μg/mL	74.01621 ± 0.4617	
	R. Hirtellus roots	methanol	125 μg/mL	73.4383 ± 0.523	252.525 μg/mL
			500 μg/mL	77.7948 ± 0.359	
		aqueous	125 μg/mL	75.0128 ± 0.532	325.3331 μg/mL
			500 μg/mL	74.6117 ± 3.829	
	ascorbic acid (standard)		100 μg/mL	98.074 ± 0.07438	70.7107 μg/mL
ABTS	*R. Hirtellus* aerial parts	chloroform	25 μg/mL	20.876 ± 4.4290	154.4 μg/mL
			800 μg/mL	59.068 ± 5.6833	
		n-hexane	25 μg/mL	1.5694 ± 0.4055	78.31 μg/mL
			800 μg/mL	52.8299 ± 5.6345	
		ethyl acetate	25 μg/mL	32.3686 ± 6.1167	67.03 μg/mL
			800 μg/mL	80.9001 ± 1.5744	

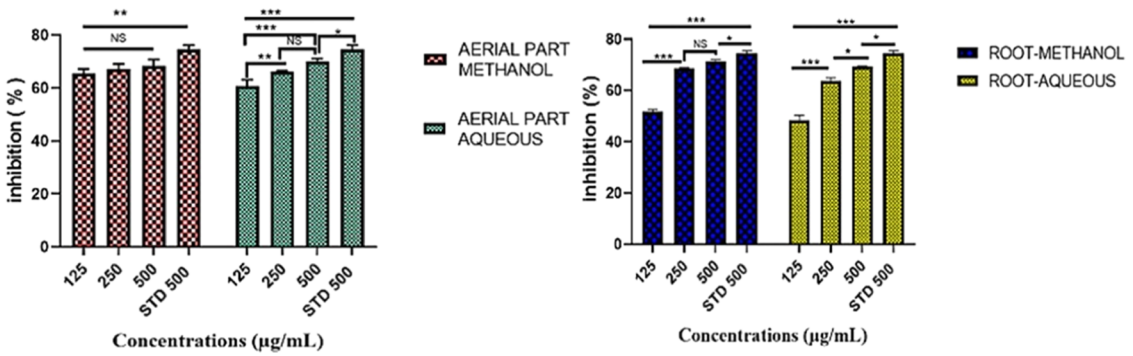

Figure 8. α-Amylase (% inhibition) through *R. hirtellus* aerial part and root extracts (methanol and aqueous) compared to the standard. The experiments were performed in triplicate, and results were expressed as mean ± SD with the significance level (NS = nonsignificant, *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).

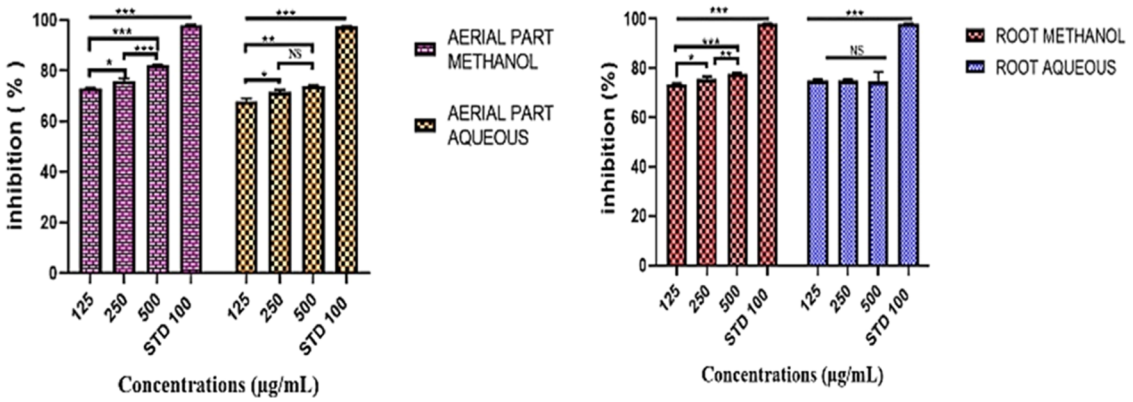

Figure 9. DPPH (% inhibition) through the *R. hirtellus* aerial part and root extracts (methanol and aqueous) compared to the standard. The experiments were performed in triplicate, and results were expressed as mean ± SD with the significance level (NS = nonsignificant, *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).

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aqueous, the concentrations are not dose-dependent and are nonsignificant compared to standard. Table 6 presents the percentage inhibition of DPPH activity with its IC_{50} values in methanolic and aqueous extracts.

Effect of Plant Crude Extracts on ABTS (In Vitro). Radical scavenging of three extracts (chloroform, *n*-hexane, and ethyl acetate) of the aerial part of the plant was assessed through ABTS assay. The highest percentage inhibition of absorbance of ABTS assay was recorded at a dose of 500 μg/mL, while the lowest was at a concentration of 125 μg/mL. In *R. hirtellus* aerial part's chloroform extract, the percent inhibition was at a concentration of 25 μg/mL (20.876 ± 4.4291%), 50 μg/mL (32.0817 ± 2.093%), 100 μg/mL (35.3848 ± 4.0559%), 200 μg/mL (43.346 ± 2.5097%), 400 μg/mL (51.8545 ± 4.1046%), and 800 μg/mL (59.0683 ± 5.6833%). In *n*-hexane extract, the % inhibition was at a concentration of 25 μg/mL (1.5694 ± 0.4055%), 50 μg/mL (15.5929 ± 1.76093%), 100 μg/mL (25.5214 ± 2.05871%), 200 μg/mL (36.7172 ± 1.51746%), 400 μg/mL (44.3328 ± 3.12245%), and 800 μg/mL (52.8299 ± 5.6346%). Additionally, the percent inhibition of ethyl acetate was at a concentration of 25 μg/mL (32.3686 ± 6.1167%), 50 μg/mL (46.3309 ± 3.26942%), 100 μg/mL (56.16 ± 4.6249%), 200 μg/mL (66.20 ± 1.89744%), 400 μg/mL (72.85 ± 2.064%) and 800 μg/mL (80.90 ± 1.5744%). Furthermore, chloroform, *n*-hexane, and ethyl acetate extracts dose-dependently inhibit the ABTS level as depicted in Figure 10 and *p*-values are mentioned in the Supplementary File (Figure S11). IC_{50} values of chloroform, *n*-hexane, and ethyl acetate are 154.4, 78.31, and 67.03 μg/mL, respectively, as mentioned in Table 6.

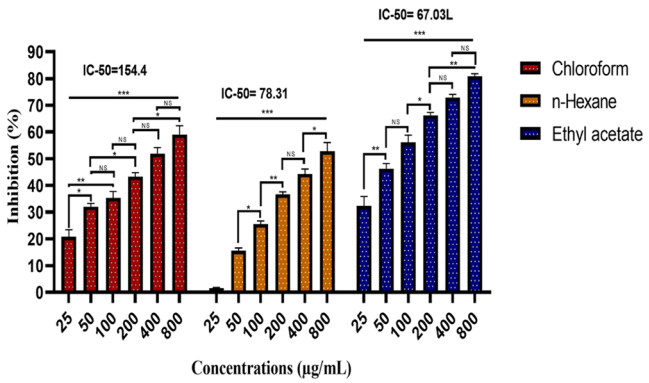

Figure 10. ABTS % inhibition through *R. hirtellus* aerial part extracts (chloroform, *n*-hexane, and ethyl acetate) at different concentrations (μg/mL). The experiments were performed in triplicate, and results were expressed as mean ± SD with the significance level (NS = nonsignificant, $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$).

DISCUSSION

Medicinal plants serve as the primary source of bioactive compounds with pharmacological activities, representing a crucial resource for both traditional and modern medicines.[16,57] Their significance lies in their high efficacy and low cost.[58] The presence of volatile compounds in the bodies of medicinal plants varies across different seasons and life stages. These compounds play crucial roles, serving to repel pests, inhibit the growth of surrounding plants, and attract pollinators.[59] A variety of techniques have been developed for the detection and isolation of bioactive compounds from plants. These techniques encompass methods such as chromatography, spectrometry, and extraction processes, enabling researchers to identify and isolate specific compounds with pharmacological significance. The GC/MS results reveal that in the three extracts of ethyl acetate, chloroform, and *n*-hexane, 14 different phytochemical compounds were identified both in *R. hirtellus* aerial parts and in roots. The presence of these phytochemical ingredients from three separate extracts was discovered in the GC/MS analysis of *R. hirtellus* aerial parts (leaves and stem) and roots as listed in Tables 1 and 2. The reported compounds belong to different classes as depicted in Tables 1 and 2. Among these compounds, two compounds have been reported with antidiabetic[52,56] activities and five have been reported with antioxidant[53,56,54,55] effects. In antioxidant compounds, 2,4-di-*tert*-butylphenol[55] is a commercially available antioxidant as shown in Tables 1 and 2. Furthermore, out of 28 compounds, 16 have been reported in other plants having antioxidant or antidiabetic activities, as shown in Supporting (S) Table 1. This discovery suggests that these chemicals might contribute to the plant's therapeutic quality.

Following the GC/MS analysis, we were interested in qualitatively checking 16 different compounds in two different extracts of plants: methanolic and aqueous. The results show that the flavonoids, sterols, glycosides, terpenoids, sterols, lactones, and lignin were present in the methanolic extract of *R. hirtellus*. Alkaloids sterols, triterpenes, glycoside, leucoanthocyanins, terpenoids, sterols, and lactones were present in aqueous extracts of *R. hirtellus*. Carbohydrates, tannins, saponins, amino acids, anthocyanins, and phlobatannins were absent in both methanolic and aqueous extracts. Alkaloids, leucoanthocyanins, and triterpenes were present in aqueous extracts but absent in the methanolic extract, while flavonoids and lignin were present in methanolic extracts but absent in the aqueous extract. These newly detected compounds in *R. hirtellus* suggest their potential in treating various ailments. These compounds have been reported in several studies from other medicinal plants.[60,61]

Flavonoids, tannins, and phenolic compounds possess different pharmacological activities as discussed by antibacterial, antiallergic, antidiabetic, anti-inflammatory, and anticancer activities.[62−64] Recently, research on carbohydrates also caught interest for diabetes treatment. Polysaccharides from guava leaves possess potential antioxidant and antidiabetic effects.[65] The results of the current study show that the flavonoids, tannins, phenolic, and RSC contents were present at different levels in these extracts of the *R. hirtellus* plant.

Many naturally occurring substances and plant extracts had previously been found to have antidiabetic properties in experimental studies.[6649] After performing the qualitative tests, we intended to know about the effect of extracts of these plants on α-amylase enzyme inhibition. α-Amylase, a key enzyme in carbohydrate digestion, plays a crucial role in managing blood glucose levels. Inhibitors of this enzyme are under investigation for their potential to regulate hyperglycemia by delaying carbohydrate digestion.[5] All of the extracts possess a dose-dependent α-amylase inhibitory effect. α-Amylase is secreted in the mouth from salivary glands and duodenum from the pancreas. Its inhibitory effect can reduce blood glucose levels by preventing the conversion of carbohydrate polymers into monomers in the gastrointestinal tract, particularly in the mouth and duodenum. This inhibitory effect of crude extract can reduce the absorption of carbohydrate monomers

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(glucose) from GIT to the blood and reduce the glucose level in the blood.[67] This α-amylase inhibitory effect might be due to the presence of different phytochemicals in the extracts. Additionally, there is no reported study on the α-amylase inhibitory effect of *R. hirtellus* aerial parts, while the results of the current study are similar to those of refs 68−70.[68−70] The free-radical-induced oxidative stress is a recognized contributor to degenerative diseases.[7] This phenomenon contributes to the development of diabetes mellitus (DM) and a multitude of other health conditions, impacting metabolic processes and overall well-being.[8] Antioxidants regulate free radicals and oxidative reactions in the body,[7] forming an integral part of defense mechanisms against various diseases, including diabetes.[10] Natural products with antioxidant properties have gained attention in diabetes research[11] due to fewer side effects and easy availability. In the current study, the DPPH and ABTS results, methanol, and aqueous extract show antioxidant activity. So, the antioxidant effect of extract can prevent pancreas damage and increase the production of insulin, which can decrease the blood glucose level. Furthermore, it has been described that aqueous is more effective than methanol in extracting antioxidant compounds.[71−73]

CONCLUSIONS

R. hirtellus revealed a diverse array of phytochemicals, including sterols, quinones, glycosides, lactones, lignin, flavonoids, alkaloids, triterpenes, terpenoids, leucoanthocyanins, TTC, TPC, TFC, reducing sugar content, and 28 other compounds. This comprehensive phytochemical profile highlights the rich chemical diversity present in the plant. The extracts from aerial parts and roots showed significant antioxidant activity, scavenging free radicals in the DPPH and ABTS assays, indicating the presence of potent antioxidant compounds. In vitro assay revealed dose-dependent antidiabetic activity in extracts of *R. hirtellus* through inhibition of α-amylase activity. This indicates the potential of *R. hirtellus* extracts as natural antidiabetic agents. The observed antioxidant and antidiabetic activities of the extracts of *R. hirtellus* suggest its potential as a source of natural antioxidants and antidiabetic agents. Further investigation into the underlying mechanisms of action is warranted to fully understand the therapeutic effects of *R. hirtellus*.

The reported plant is a novel subject for research, holding immense potential for exploration in various fields such as cancer, hormonal diseases, immune diseases, respiratory diseases, and more. Its molecular mechanisms offer opportunities for studying metabolic disorders, mitochondrial diseases, and other conditions. Hence, we strongly recommend further exploration of this plant through different in vitro and in vivo experiments for the mentioned diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08631.

Figure S1: *p*-value of biological activities according to their concentration; Table S1: compounds in *R. hirtellus* aerial and roots parts with their direct and in-direct antioxidant and antidiabetic effect (PDF)

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Author Contributions

○H.I. and B.A. contributed equally to this work. BA designed the main idea. HI, NZ, and BA performed experiments and prepared the first draft of the paper. H.I., K.A., and A.M. helped in GCMS analysis. B.A., L.J., A.A., S.U.R., M.A.A., M.A.E., and M.Y.Z. revised the manuscript and prepared the final draft. I.K. performed and analyzed the ABTS and carbohydrate tests. H.I., B.A., M.Y.Z., and M.A.E. revised the final revision of the manuscript. M.A.E. designed a new graphical abstract.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

WHO=World Health Organization; ROS=reactive oxygen species; TTC=total tannin content; TPC=total phenolic content; TFC=total flavonoid content; RSC=reducing sugar content; GC/MS=gas chromatography mass spectrophotometer; ABTS=2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate); DPPH=diphenyl-pieryl hydrazyl

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